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DESIGN OF AN INSECT CUTICLE ASSOCIATED WITH OSMOREGULATION: THE POROUS PLATES OF CHLORIDE CELLS IN A MAYFLY NYMPH

Key words: Insect, Ephemeroptera, cuticle, ultrastructure, osmoregulation, chloride.

ABSTRACT. In mayfly nymphs of the genus *Coloburiscoides*, cell complexes with an osmoregulatory function (so-called chloride cells) are found in the integuments of the oral gills, the abdominal gills and gill filaments, the coxae and the thoracic sternites. The cuticle overlying each cell complex is a rigid circular plate which is known to be porous to colloidal lanthanum suspensions. The present study shows that the plate is composed only of the cuticulin and dense layers of the epicuticle. Both layers have substructures built of subunits on almost perfect hexagonal lattices. The lattice spacings are 53 and 9·5 nm for the dense layer and the cuticulin layer respectively. During moulting the apical plasma membrane of the chloride cell remains adpressed to the old porous plate. The new porous plate is formed from a new chloride cell which intrudes from the base of the integument. Throughout the moult small pores persist in the new and otherwise continuous cuticle to allow continuity of the cytoplasm of the apical and basal portions of the old chloride cell. It is thought that this phenomenon allows osmoregulatory function of the chloride cell complex to be maintained during the moult.

Introduction

Special structures consisting of single cells or cell complexes together with overlying discs of modified cuticle have been described within the integuments of mayfly nymphs from several families (Wichard and Komnick, 1971; Komnick and Abel, 1971; Wichard *et al.*, 1972). By use of histochemical and radioactive labelling techniques the cell complexes have been shown to absorb salts from the aquatic environment and have, therefore, been assumed to be involved in osmoregulation (Komnick *et al.*, 1972). Because of their structural and functional similarities to chloride cells in teleost gills (Bierther, 1970) they have been named ephemerid chloride cells (Wichard and Komnick, 1971).

In this paper we describe some fine details of the cuticular plates, known as porous plates (Wichard *et al.*, 1972) and their underlying chloride cells and discuss the morphological basis of their permeable nature, as demonstrated previously by use of colloidal lanthanum hydroxide (Komnick and Abel, 1971).

Materials and Methods

The chloride cell complexes studied were obtained from nymphs of an undescribed species of mayfly belonging to the genus *Coloburiscoides* Lestage (Ephemeroptera, Oligoneuriidae). Specimens for study were collected from Big Pat's Creek, a small tributary of the Yarra River, at East Warburton, Victoria, Australia. Nymphs were taken from amongst tree roots which grow into the stream and were returned live to the laboratory.

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For transmission electron microscopy (TEM), gill filaments and small pieces of integument were fixed in 2·5% glutaraldehyde in 0·1 M cacodylate buffer containing 0·15 M sucrose at pH 7·2 for 2 hr at room temperature. Specimens were washed in several changes of 0·1 M cacodylate buffer with 0·15 M sucrose at pH 7·2 for a total of 2 hr at room temperature and post-fixed for 2 hr in 1% osmium tetroxide in 0·05 M cacodylate buffer at pH 7·2. The samples were dehydrated through a graded ethanol series and propylene oxide, then embedded in Spurr's Epoxy resin and sectioned with a Reichert OMU 3 ultramicrotome using diamond knives. Sections were stained with uranyl acetate and lead citrate and examined in a JEOL 100C TEM.

For scanning electron microscopy (SEM), specimens were fixed in the above glutaraldehyde solution, dehydrated in alcohol, transferred to amyl acetate and then critical point dried using carbon dioxide. Dried specimens were coated with a gold layer approximately 20 nm thick and examined in a JEOL JSM 35C SEM. To reduce con-

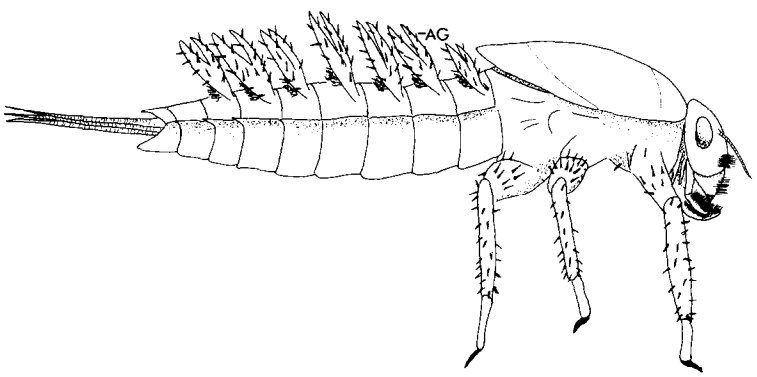

Fig. 1. A *Coloburiscoides* nymph showing the location of the abdominal gills (AG).

Fig. 2. SEM of the dorsal abdomen showing paired, segmentally arranged gills. ×10.

Fig. 3. Ventral view of a single gill showing the tuft of gill filaments. Chloride cells are found on the cuticle surrounding the base of the tuft and extend up each filament to its point of bifurcation (arrows). ×44.

Fig. 6. Dorsal view of an abdominal gill showing the area (right of dotted line) in which chloride cells are found. ×72.

Fig. 7. Porous plates of chloride cell complexes of the coniform type distributed over the surface of the basal region of a gill filament. The wrinkled appearance of the cuticle between plates is partly a shrinkage artefact produced during critical point drying. ×1280.

Fig. 8. Porous plates on the ventral gill cuticle. Both coniform (co) and caviform (ca) types are present. ×2000.

Fig. 9. Porous plates of the coniform type only, on the thoracic sternite cuticle. ×3200.

Fig. 10. Single porous plate of the coniform type showing bacteria attached to the plate by fine filaments. Pores on the rim of the plate are indicated by arrows. ×12,000.

Fig. 11. Oblique view of the plate of a single coniform cell complex, showing three pores (arrows) on the surrounding raised rim. ×13,000.


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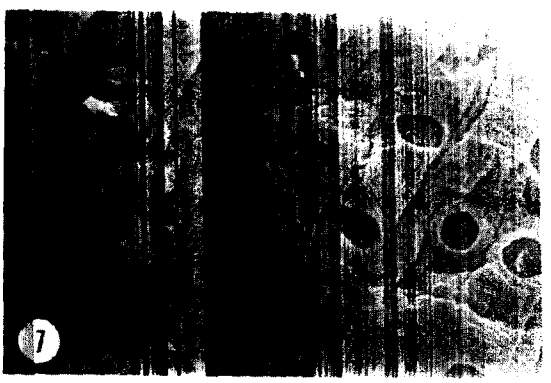
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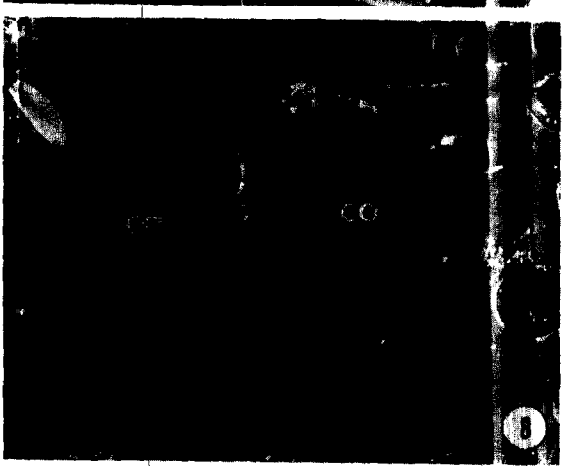
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tamination on the cuticle surface from the aquatic environment (bacteria, etc.) some specimens were given a brief ultrasonic treatment in mild detergent before fixation.

Results

Distribution of chloride cells and porous plates

Coloburiscoides nymphs have a pair of gill structures on each of the abdominal segments 1–7 (Fig. 1). Each structure is heavily sclerotized, spiny, and horseshoe-shaped dorsally, and will be referred to as the gill. Attached ventrally to the gill are branched filamentous structures, which will be referred to as the gill filaments (Figs. 2, 3). Presumably the gills are used as anchoring devices by the nymphs, which live under stones in fast currents. The filaments have a respiratory function (Wichard and Komnick, 1971).

In addition, *Coloburiscoides* nymphs have two pairs of finger-like oral gills. These are attached to the bases of the maxillary and labial palps (Figs. 4, 5). Oral gills have previously been described from the nymphs of a number of species of oligoneuriid mayflies (e.g. Edmunds *et al.*, 1976). Their presence on nymphs of *Coloburiscoides* has previously been noted (Edmunds, 1957), although they have not been included in descriptions of the nymphs.

Porous plates associated with chloride cell complexes are most abundant on the

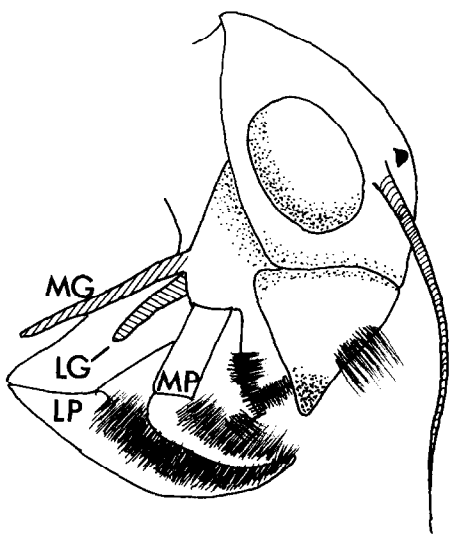

Fig. 4. Lateral diagrammatic view of the head of a *Coloburiscoides* nymph showing the position of the oral gills. The gills are shaded. MP, maxillary palp; LP, labial palp; MG, maxillary gill; LG, labial gill.

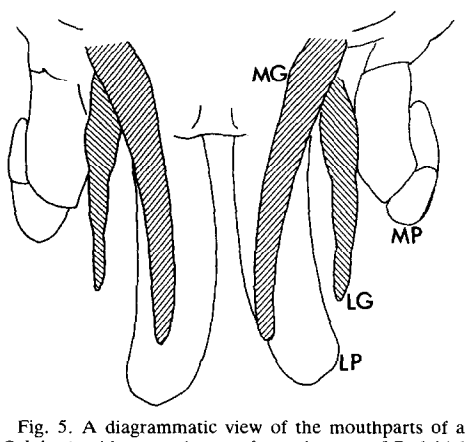

Fig. 5. A diagrammatic view of the mouthparts of a *Coloburiscoides* nymph seen from the rear. LP, labial palp; LG, labial gill; MP, maxillary palp; MG, maxillary gill.

Fig. 12. Higher magnification of part of the plate shown in Fig. 11, with detail of the raised plug of material within the peripheral pores. ×48,000.

Fig. 13. Porous plate of the caviform type with its associated pore. ×16,000.

Fig. 14. Tranverse section of a coniform chloride cell complex, showing the porous plate cuticle (p). Central cell (C) is surrounded by two layers of cells (A, B). Neighbouring cells are joined in the apical region by belt desmosomes (bd) and septate junctions (sj). tr, tracheole; b, bacteria. ×17,000.


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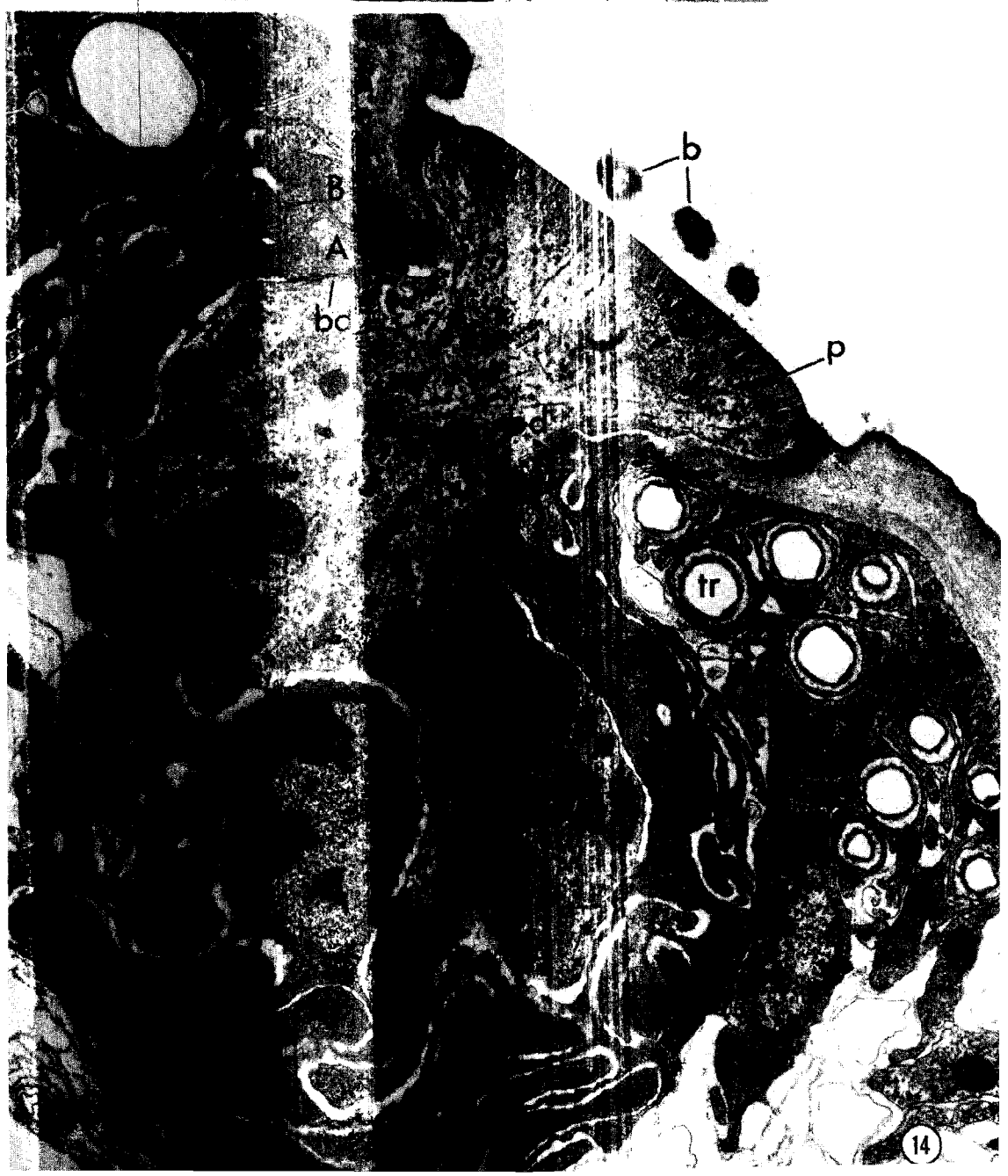
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oral gills and on the abdominal gills and filaments. They are also found on the coxae and the thoracic sternites.

SEM of the porous plates

On the abdominal gills (Fig. 2), the plates are present on the ventral surface, on a well-defined area surrounding the base of each tuft of gill filaments (Figs 3, 8) and extend up each filament to its point of bifurcation (arrows in Fig. 3; Fig. 7). The opposite dorsal surface of the gill has a similar distribution of plates in the basal region (Fig. 6).

SEM reveals porous plates of two distinct types, which reflect two types of underlying chloride cell or cell complexes similar to those described previously by Wichard *et al.* (1972) and named by them coniform and caviform respectively (Figs. 7–13). Both types are found together at the abdominal gill bases (Fig. 8), whereas on the abdominal gill filaments, the coxae and the thoracic sternites (Fig. 9) only the coniform type is present.

The cuticle of the coniform plate is smooth centrally with a slightly raised rim containing up to four minute pores, each filled with a plug of material (Figs. 10–12). The diameter of each of these pores is approximately 0·2 μm. Each coniform plate has a diameter in the range 4–6 μm and is apparently more rigid than the surrounding undifferentiated cuticle, as evidenced by its comparative resistance to wrinkling during specimen preparation (Fig. 7).

The plate of the caviform chloride cell is smaller than its counterpart, being approximately 1 μm in diameter, and is below the level of the surrounding cuticle (Fig. 13). Associated with the plate of each caviform cell is a single pore approximately 0·35 μm in diameter, also filled with a raised plug of material (Fig. 13).

Despite the ultrasonic washing pretreatment used to remove surface contamination, large numbers of bacteria remain firmly attached to the plates (mainly those of the coniform cells) by means of filaments (Figs. 9, 10).

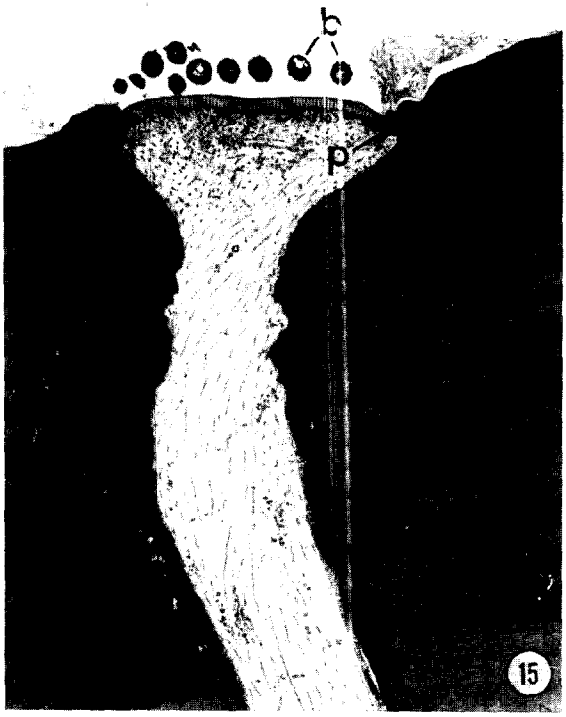
TEM of the porous plates and chloride cells

(a) *Coniform type.* The internal structure of the coniform chloride cells is similar in most respects to that described previously in nine mayfly species (Wichard *et al.*, 1972). Underlying the cuticular plate is a central cell enveloped by at least two additional cellular layers, which together comprise the chloride cell complex (Fig. 14). The apical portion of the central cell is coniform, the neighbouring undifferentiated cuticle causing a restriction in the cell approximately 1·5 μm wide and 1·5 μm below the cuticular surface. The apical plasma membrane of the central cell is formed into microvilli. The tip of each microvillus possesses an electron-dense internal coating known as a plasma membrane plaque (Fig. 16) (Locke and Huie, 1979). Numerous microtubules, orientated baso-apically, are found in the cytoplasm of the apical cone. At the ex-

Fig. 15. Transverse section of the apical part of the central chloride cell (coniform type) passing through a channel in the thick dorsal cuticle of the gill. b, bacteria; p, peripheral pore. ×10,000.

Fig. 16. Higher magnification of the pore shown in Fig. 15, showing the plume of fine fibrous material emanating from the tip of the electron-dense plug. Arrow indicates lateral hemidesmosome attachment of the cell to the cuticle. b, bacteria; mv, microvilli. ×32,000. *Insert* shows detail of the porous plate cuticle. Note vertical striation in the cuticulin layer (cu) and horizontal beading of the vertical bars in the dense layer (d). Arrows indicate microvilli. ×82,500.

Fig. 17. Near-tangential section through the porous plate of a coniform cell complex, showing the apical cytoplasm of the central cell (C) with its microvilli (mv), the dense layer (d) and the cuticulin layer (cu). Arrows indicate transverse sections at different levels through three of the peripheral pores. ×21,000.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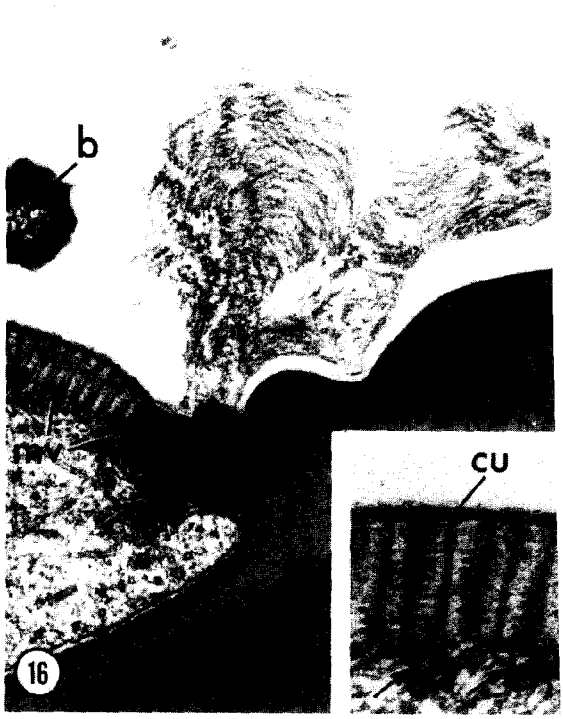
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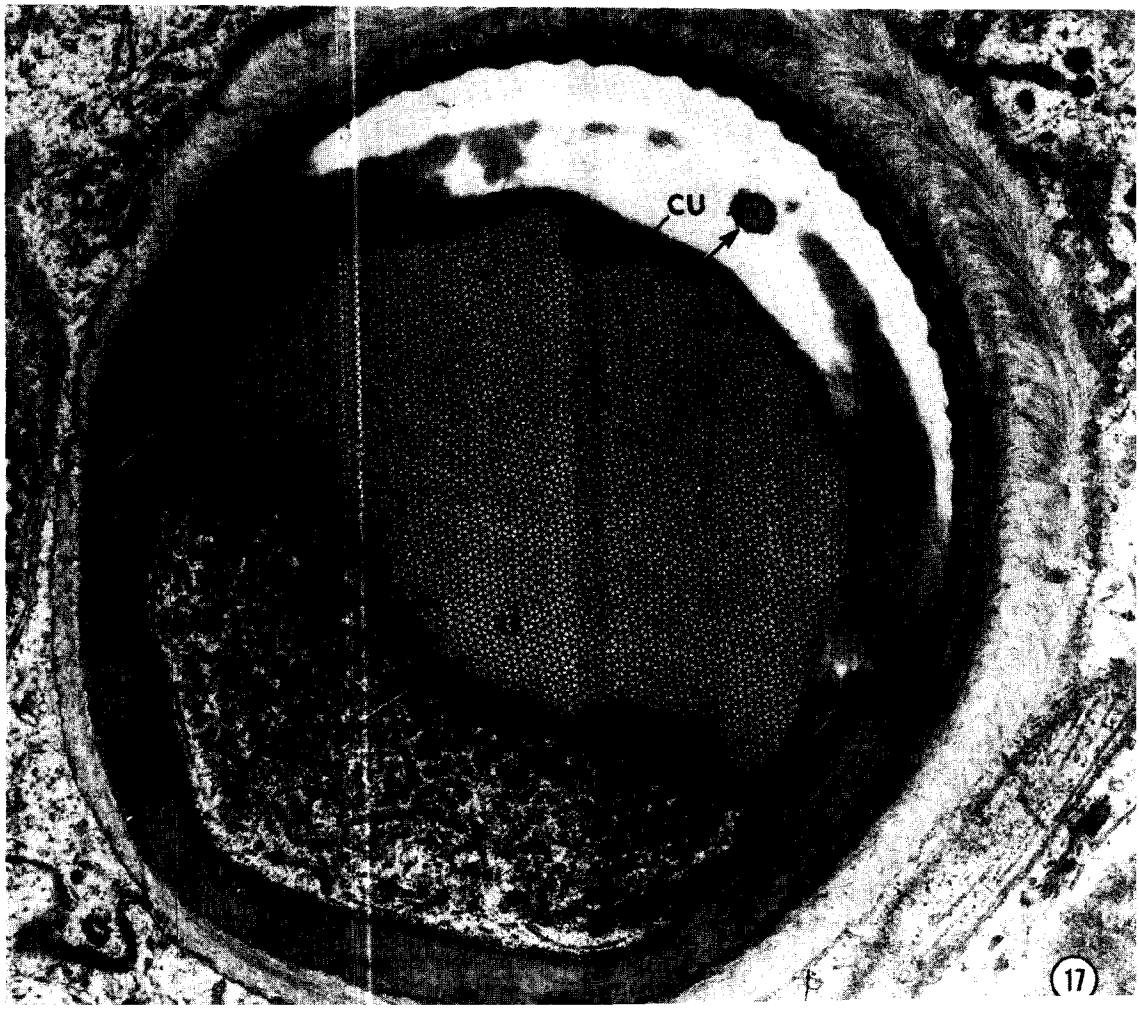
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treme lateral margins of the central cell, the 'microvillus' is probably a continuous raised rim, forming a hemidesmosome attachment with the surrounding cuticle. Microfilaments are attached at this point to the internal dense layer of the rim of plasma membrane (Fig. 16). The apical portion of only the central cell projects on to the interior face of the porous plate. The surrounding cells project apically only to the level of the interior of the neighbouring, thicker, normal cuticle. In the gill filaments, this normal cuticle is very thin (average 0·6 μm in thickness). The other areas of integument examined by TEM were on the gill proper, at the base of the gill filaments and on the opposite, dorsal surface of the base of the gill. In these areas, the normal cuticle is much thicker (average 5 and 15 μm respectively). Here a process of the central cell passes apically through a channel approximately 2 μm wide in the normal cuticle to make contact with the thin cuticle of the porous plate (Fig. 15). The cells surrounding the central cell are attached to one another and to the central cell by belt desmosomes and septate junctions only near their apices (Fig. 14). Basal to the attachment region there are electron-lucent extracellular spaces of variable width and complex basal infolding and intercellular interdigitation. The surrounding cells contain numerous mitochondria and possess a higher overall electron density of their cytoplasm than the central cell.

The cuticle of the porous plate consists only of epicuticle which is subdivided into two distinct layers. For consistency of nomenclature we will call these the cuticulin and the dense layer, although their homologies with the corresponding layers in normal insect cuticles are not certain.

The cuticulin layer has a total thickness of 15–20 nm and is in turn subdivided into a variable number of light and dark layers (Fig. 16, insert). There is an outermost electron-dense layer approximately 5 nm thick and two or three inner dense layers approximately 2·5 nm thick separated by electron-lucent layers also about 2·5 nm thick. The electron-dense layers show clear regular vertical striations giving the impression of a porous substructure.

The thickness of the dense layer of the epicuticle differs depending on the location of the porous plates. Within the gill filaments the thickness is approximately 100 nm (Fig. 14), whereas within the dorsal cuticle of the gill proper it is approximately 200 nm thick (Fig. 16). The dense layer also has a substructure of vertical light and dark bands, but this is much coarser than in the cuticulin layer. These vertical bands sometimes exhibit a beaded appearance (Fig. 16, insert), possibly indicating a regular horizontal subdivision into laminae.

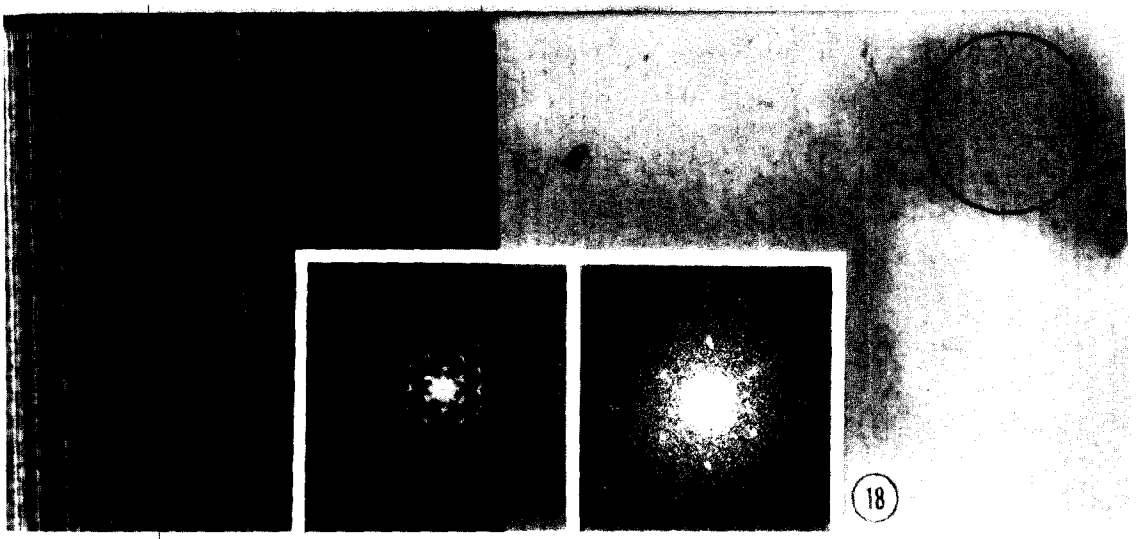
When this cuticle is sectioned tangentially to the surface, both the cuticulin and the dense layers are seen to have substructures built on two-dimensional hexagonal lattices (Figs. 17–20). In the dense layer (Fig. 19), the pattern (in the plane of section) is composed of electron-dense circular profiles approximately 26 nm diameter arranged on the hexagonal lattice with a spacing, measured from optical diffractograms (Fig. 18,

Fig. 18. Tangential section through the porous plate of a coniform cell passing through the dense layer (left) and cuticulin layer (right). ×60,000. Optical diffractograms of the areas enclosed by the left and right circles are shown in the left and right inserts respectively.

Fig. 19. Detail of the hexagonal pattern of the dense layer. Outlines show electron-dense circular profiles connected to nearest neighbours by electron-dense struts. ×125,000.

Fig. 20. Fine structure of the cuticulin layer seen in tangential section is one of electron-lucent, hexagonally packed subunits in an electron-dense matrix. ×250,000.

Fig. 21. Transverse section of a chloride cell complex of the caviform type in the dorsal gill cuticle. The central cell has a large lumen with inwardly projecting fine microvilli. ×7600.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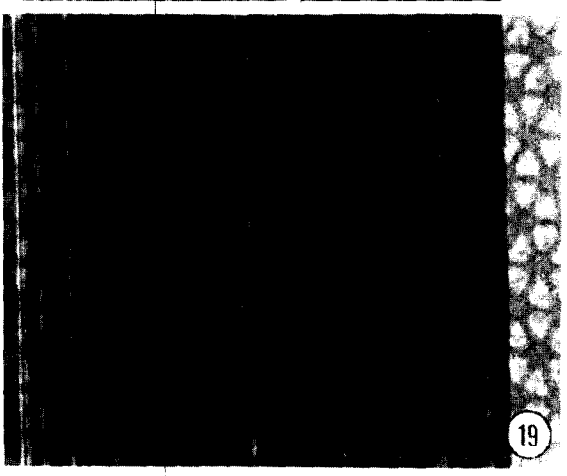
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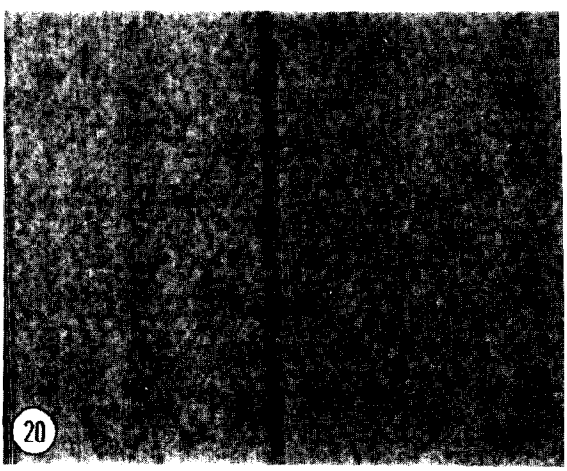
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insert), of 53 nm. Nearest-neighbour subunits are connected to one another by electron-dense struts 5–6 nm thick. The remaining electron-lucent parts of the structure have the appearance of interlaced rosettes of triangular profiles. At relatively low magnification (Fig. 17), the long-range order of the hexagonal pattern is seen to have many dislocations. In the cuticulin layer (Fig. 20), the substructure is one of electron-lucent, hexagonally packed, circular profiles in an electron-dense matrix. The spacing of the subunits on the hexagonal lattice, as measured from optical diffractograms (Fig. 18, insert), is approximately 9·5 nm.

The pores surrounding the porous plate, described earlier from SEM (Figs 10–12), have also been observed in transverse (Figs. 15, 16) and tangential (Fig. 17) sections of the cuticle. The rim of cuticle in which the pore lies is normal epicuticle intruding into the region above the lateral apical margin of the central chloride cell (Figs. 15, 16). The pore itself is 0·2–0·25 μm in diameter and is filled with electron-dense material which projects a short distance above the surface of the surrounding cuticle to form a small papilla (Fig. 16). Exuding from the papilla is a plume of electron-dense, fine, fibrous material which extends laterally, mainly in the direction of the normal cuticle where it forms a thin, irregular and apparently continuous membrane (Fig. 15).

Bacteria attached to the porous plates referred to earlier from SEM (Figs. 9, 10) are also seen in sections (Figs. 14–16). Their filamentous attachments are not as clearly visible in sections as they are in SEM.

(b) *Caviform type.* Wichard *et al.* (1972) found this type in 12 of the 16 species they examined and described it as a single cell, as distinct from a complex of several cells found in the coniform type. In contrast to their observations, we have found that in *Coloburiscoides*, this type is a complex of cells, although the structure of the central cell and the porous plate are very similar to that previously described (Wichard *et al.*, 1972).

The central cell contains a large lumen with numerous inwardly projecting, fine, irregular and branched processes or microvilli (Fig. 21). The remainder of the lumen of the central cell is filled with sparse, fine fibrous material (Figs. 22, 23). The only parts of the central cell that reach to the apical porous plate are fine processes at the extreme lateral margins and these make contact with dense areas of epicuticle from the neighbouring normal cuticle (Fig. 22). Belt desmosomes and septate junctions in the apical region join the central cell to the two layers of surrounding cells (Fig. 22).

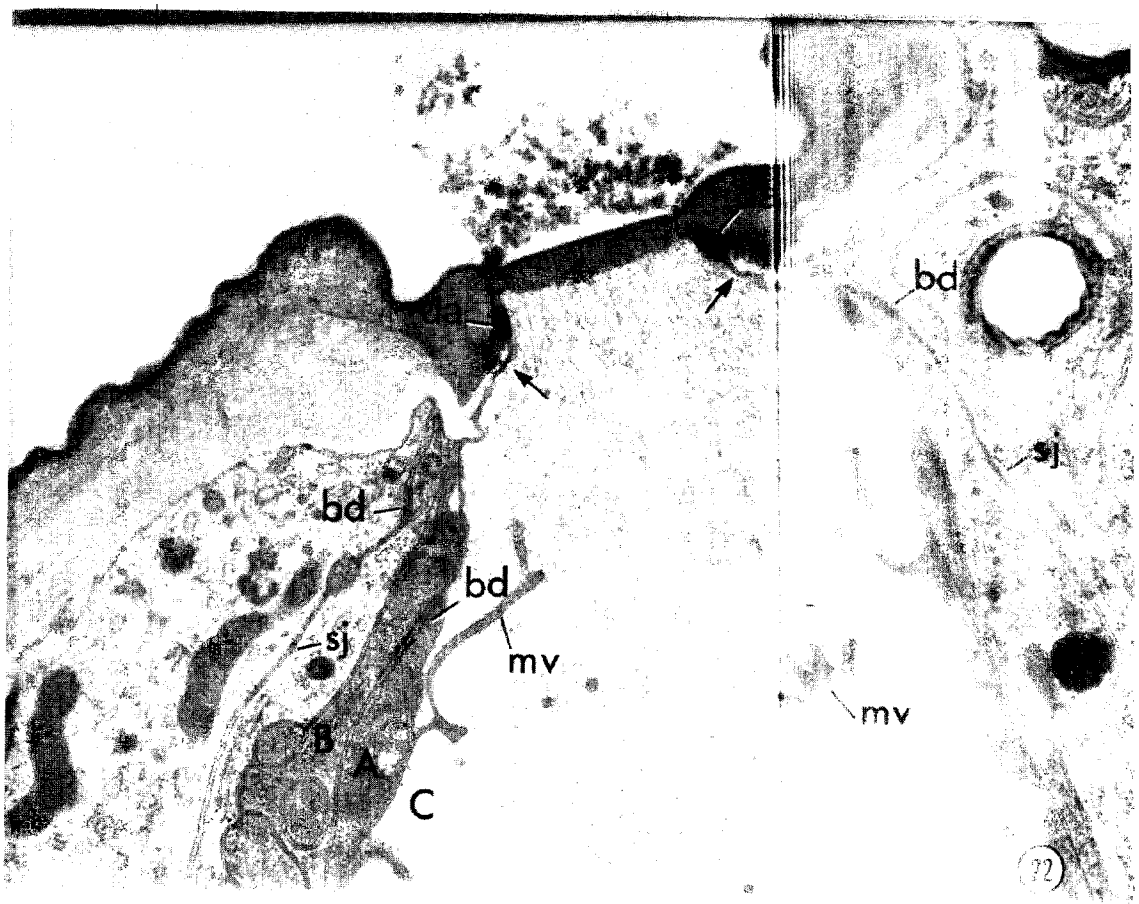
Most features of the cuticle of the caviform porous plate are identical to those of

Fig. 22. Transverse section of the apical region of a caviform chloride cell complex. Fine projections of the central cell (arrows) pass apically to join with dense areas (da) in the surrounding cuticle. The central cell (C) is surrounded by at least two layers of cells (A, B). Cells are joined apically by belt desmosomes (bd) and septate junctions (sj). mv, microvilli. ×20,000.

Fig. 23. Transverse section of a caviform porous plate with its associated pore. ×50,000.

Fig. 24. Transverse section of coniform cell complexes during a moult. New epicuticle (epi) is being secreted beneath the old cuticle. Apical cytoplasm of the old coniform cell (C1) remains adpressed to the old cuticle of the porous plate (pl) whilst neighbouring undifferentiated epidermal cells have retracted from the old cuticle to form the new one. Pores providing cytoplasmic bridges between the apical and basal parts of C1 are out of the plane of this section. C2, new coniform cell; A, B, surrounding cells; mg, moulting granules. ×25,000.

Fig. 25. Similar to Fig. 24, but sectioned through a pore (arrow) in the new porous plate (p2) and showing a cytoplasmic bridge joining the apical and basal portions of C1. Other labelling as for Fig. 24. ×25,000.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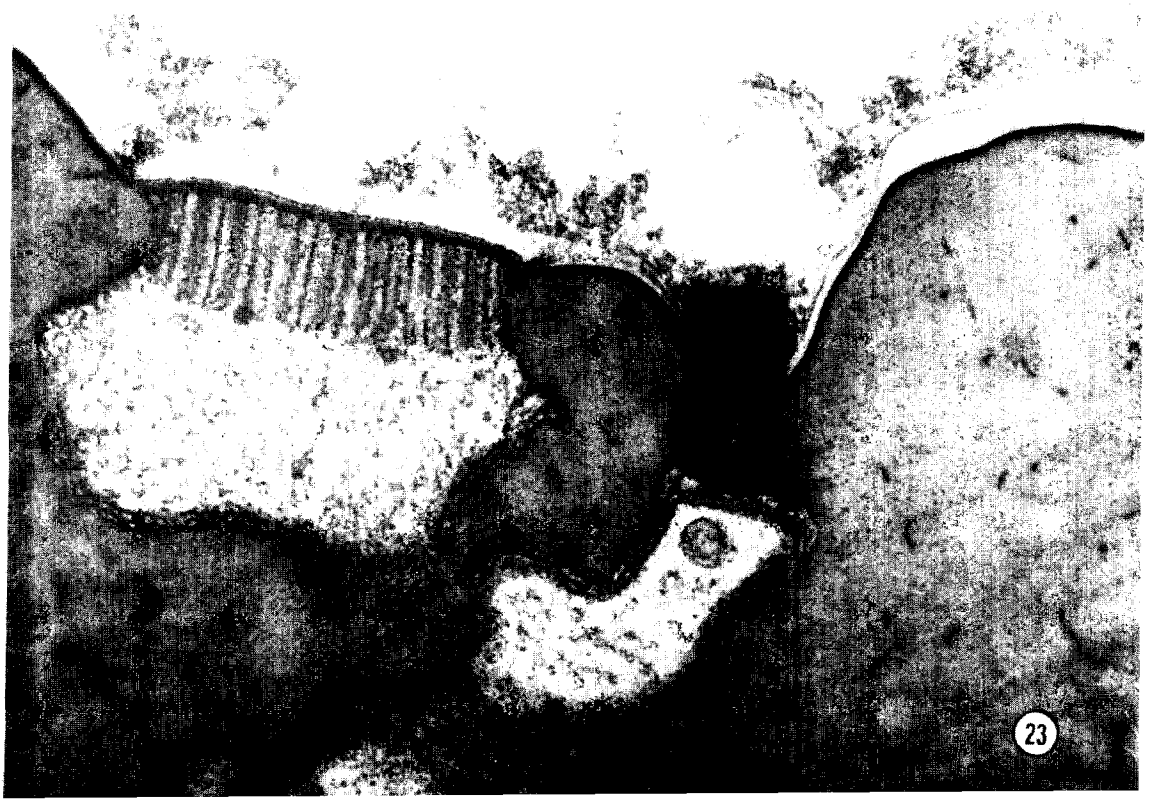
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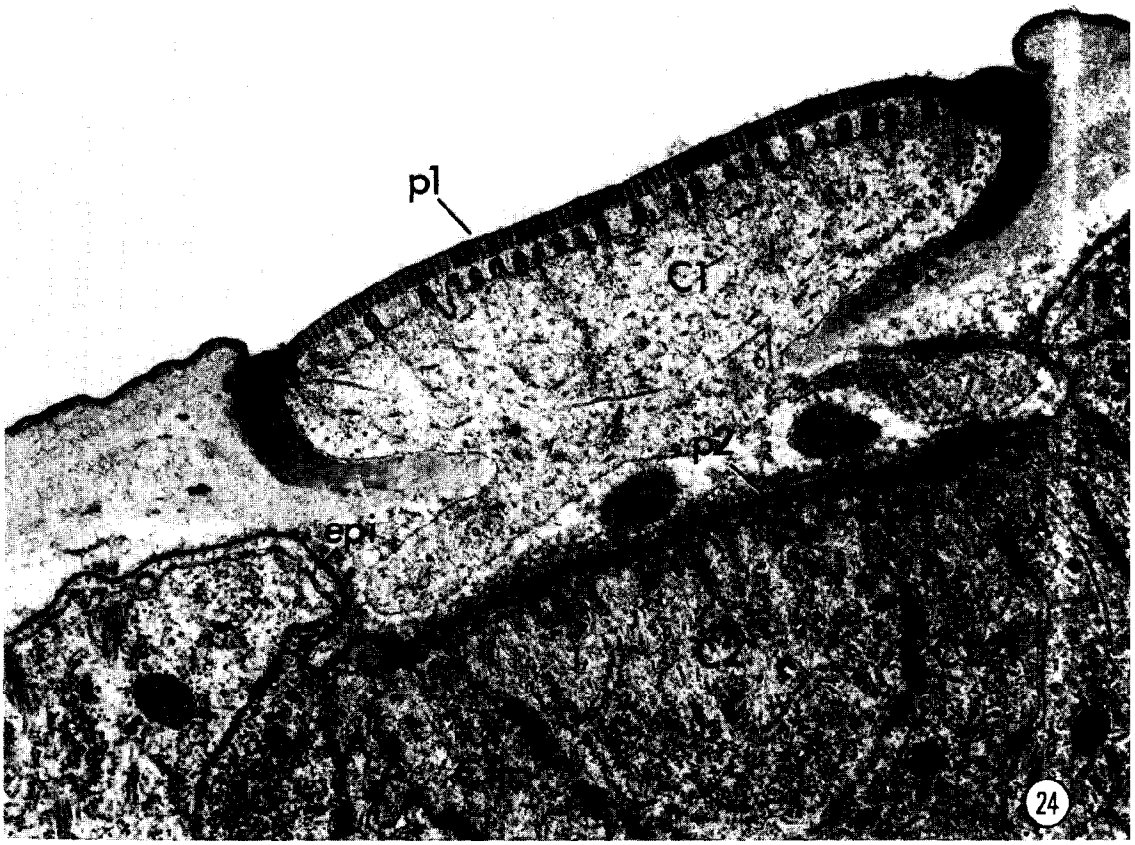
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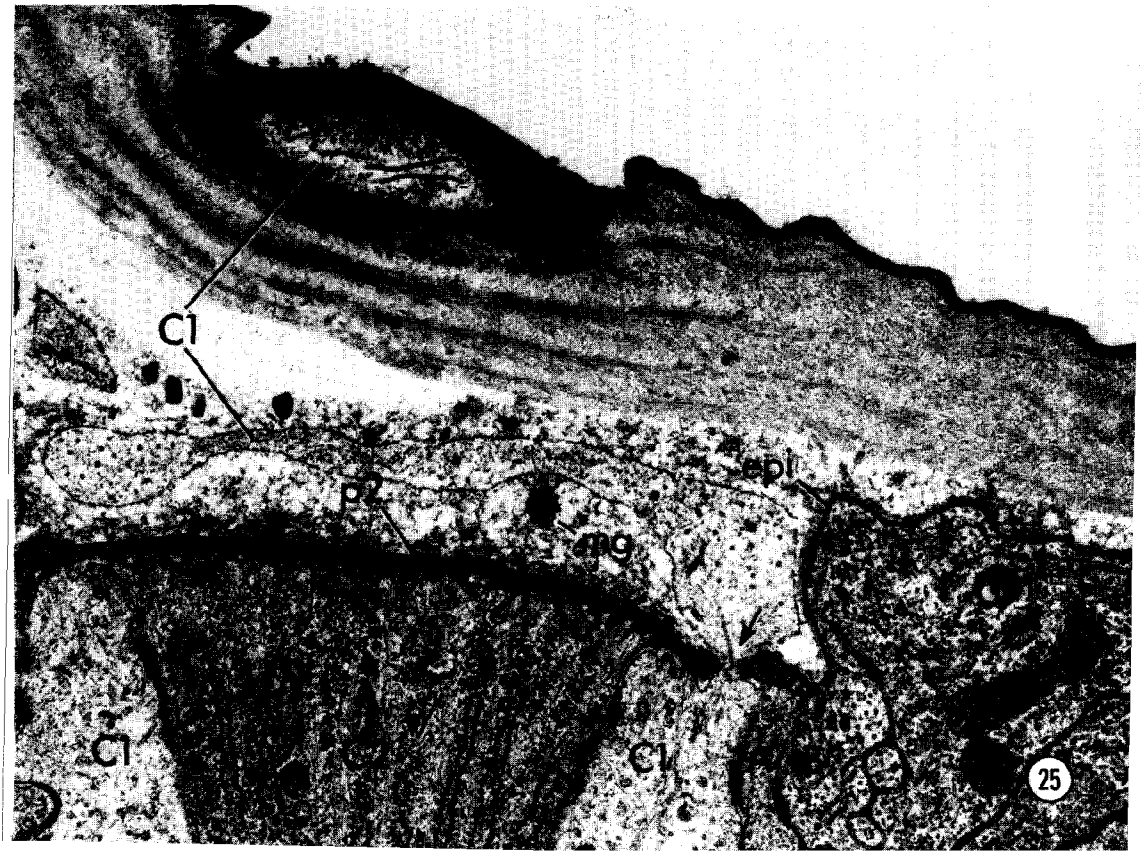
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the coniform type. The only obvious difference is in the thickness of the dense layer, being 0·25–0·3 μm in the caviform type (Figs. 20–22) compared with 0·1–0·2 μm in the coniform type (Figs. 14, 16).

A transverse section of a caviform plate which includes its associated single pore is shown in Fig. 23 (compare with SEM, Fig. 13). As with the pores of the coniform type, it is filled with electron-dense material which forms a raised papilla, with fine fibrous material emanating from the tip.

The cuticle and cell complexes during moulting

In *Coloburiscoides*, the moulting process in regions of the integument containing chloride cell complexes is similar to that described previously in other species of mayfly nymphs (Komnick and Abel, 1971; Komnick *et al.*, 1972). Formation of the undifferentiated cuticle surrounding the porous plates occurs in the usual way. The epidermal cells retract from the old cuticle, moulting fluid is secreted into the exuvial space and a new cuticle is formed on the apical face of the epidermal cells. The cuticle of the porous plate, however, is formed not from the existing chloride cell, but from a replacement cell which intrudes from the basal side of the integument. This phenomenon is illustrated in Figs. 24 and 25, where the epicuticle and porous plate of the new cuticle are already being secreted. The new epicuticle is almost but not quite continuous between the porous plate and the surrounding undifferentiated cuticle. In favourable sections (Fig. 25) small pores can be seen at the edges of the porous plates, allowing bridges of cytoplasm joining basal and apical portions of the old chloride cell to be maintained throughout the moult. At ecdysis the apical portion of the old chloride cell is pinched off and shed with the old cuticle. The scars of the cytoplasmic bridges persist in the fully formed cuticle as the pores on the periphery of the plate described earlier in SEMs and TEMs (Figs. 10–13, 15–17). The plume of material which is often seen emanating from the pore in fully formed cuticle (Figs. 15, 16) is therefore likely to be the remnant of the apical cytoplasm of the old chloride cell. The fate of the basal portion of the old chloride cell after ecdysis is unknown.

Discussion

Structure and permeability of the porous plates

The porous plate is composed only of epicuticle, subdivided into an outer cuticulin and an inner dense layer, both of which show regular substructures built on hexagonal lattices. The cuticulin layer has not been visualized in previous studies (e.g. Komnick and Abel, 1971; Wichard *et al.*, 1972) where the basis for the permeability of the plates was assumed to be the large pores in the underlying dense layer. Demonstration of the presence of a cuticulin layer overlying the dense layer leads to the conclusion that the permeability properties of the porous plate are more likely to be governed by the cuticulin layer than the dense layer.

It is interesting to note that regular substructure within the cuticulin layer of insects or other arthropods has never before been seen with such clarity except in, (1) the tracheal envelope (Locke, 1966, 1976, 1982) where the tracheal lining remains permeable to water, and (2) the pre-ecdysial cuticulin in lepidopteran (*Calpodes*) larvae (Locke, 1966) where the substructure was thought to reflect the presence of minute pores which allowed selective uptake of the products of digestion of the previous cuticle.

The actual pore size in the cuticulin of the porous plate is impossible to measure from electron micrographs. The *en face* view demonstrates electron-lucent subunits with a diameter a little less than their spacing (9·5 nm), but the real pore may lie within these subunits and be much smaller, as is the pore within a subunit of similar size found in the gap junction (Peracchia, 1980). However, the pores in the cuticulin of the porous plate must be larger than those of the gap junction because the former has been shown to be permeable to colloidal lanthanum particles (Komnick and Abel, 1971) whereas the latter are impermeable (Peracchia, 1980).

The dense layer has, in some cuticles, been shown to be laminated horizontally or to possess other types of substructure (see Filshie, 1982). However, the almost perfectly crystalline substructure recorded in the porous plate of this and other species of

mayfly nymph (Wichard *et al.*, 1972), is so far unique. As well as being porous (Komnick and Abel, 1971), it must also be quite stiff because of its demonstrated resistance to deformation during preparation for SEM. The evolution of such a regular substructure may be a response to the need for the dual properties of rigidity and permeability.

Chloride cell complexes and moulting

The observations that the porous plate cuticle of successive nymphal instars is formed by replacement cells which migrate from the basal layer of the integument and that continuity persists between the apical and basal cytoplasm of the old chloride cell throughout the moult (from apolysis to ecdysis) suggests that osmoregulatory function of the chloride cell complex is maintained throughout this period.

Our observations of moulting *Coloburiscoides* nymphs also support the homology of the epicuticular layers of the porous plate with those of the surrounding undifferentiated cuticle. Komnick and Abel (1971) suggested that the porous plate represented a differentiation of the endocuticle only, but our observations demonstrate that secretion of the undifferentiated epicuticle occurs in synchrony with that of the porous plate (Fig. 25). Also, all other evidence from electron microscopy suggests that the cuticulin is universally present in arthropods (Filshie, 1982) and forms a complete envelope for their bodies (Locke, 1982).

Association of bacteria with chloride cells

We have demonstrated, by their resistance to physical removal during preparation for SEM, that surface bacteria are firmly attached to the porous plates.

It is probable that the bacteria are utilizing materials released by the chloride cell complexes. The most likely materials are ammonium and bicarbonate ions which are released as byproducts of the uptake of sodium and chloride in some aquatic insects (Keynes, 1973). It is also possible, but less likely, that the bacteria are utilizing sodium and chloride ions and amino acids that are 'leaked' during the pumping process or passively released periodically to regulate cell size (Hoffmann, 1978).

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